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Cell attachment to frozen sections of injured adult mouse brain: effects of tenascin antibody and lectin perturbation of wound-related extracellular matrix molecules

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Abstract

Previous studies describing the use of cryoculture methods have focused on the efficacy of the method for studying neuron attachment and neurite outgrowth on intact sections of nerve, and rodent and even human brain. The cryoculture method has shown promise for determining the presence of cell attachment- and neurite-growth-inhibiting molecules in such specimens, and some studies have also attempted to neutralize such molecules with antibodies to myelin inhibitory proteins, nerve growth factor, or factors present in conditioned media that may counteract the repulsiveness of some of these molecules preserved in sections of, for example, myelinated nerves or adult brain white matter. The present study describes the novel use of lesioned central nervous system cryocultures as substrates for investigating the attachment of embryonic neurons and PC12 cells. In addition to demonstrating the use of this novel scar substrate to extend previous 'scar-in-a-dish' models (David et al. (1990) Neuron, 5: 463–469; Rudge and Silver (1990) J. Neurosci., 10: 3594–3603; Rudge et al. (1989) Exp. Neurol., 103: 1–16), the present study also describes antibody and lectin perturbations of putative inhibitory molecules that result in an enhanced attachment of cells to cryosection cultures of brain and spinal cord wounds.

Keywords: Cryoculture; Brain wound; In vitro bioassay; Antibody perturbation; Lectin perturbation

1. Introduction

Since injured adult central nervous system (CNS) neurons can grow and extend process when provided with favorable substrates (e.g. peripheral nerve) (Benfey and Aguayo, 1982; David and Aguayo, 1981), and because injured peripheral nervous system (PNS) neurons, which normally demonstrate abundant regenerative growth, show retarded process extension in the injured CNS (for review see Schwab et al. (1993)), much attention has been focused on growth inhibitory factors within the lesioned adult CNS that might contribute to the poor regenerative growth seen after injury. It has been known for some time that normal CNS white matter is an unfavorable environment for attachment and process extension for many different cell types (Crutcher, 1989; Sagot et al., 1991; Watanabe and Murakami, 1989; Savio and Schwab, 1989), and in recent years factors contributing to this white matter inhibition have been extensively characterized and, in culture assays, neutralized with antibodies (Schwab and Caroni, 1988; Schwab and Thoenen, 1985). In addition to CNS white matter, it is now clear that lesioned gray matter has components that can be inhibitory to regenerating neurites. It has long been thought that hypertrophic astrocytes, present in the injured CNS, form a physical barrier that hampers regenerating neurites (Reier and Houle, 1988; Reier et al., 1983). In addition to this physical barrier, there have recently been described several extracellular matrix (ECM) molecules associated with astrocytes in and around CNS lesions that may negatively affect neuritic growth (Laywell and Steindler, 1991; Laywell et al., 1992; McKeon et al., 1991). Interestingly, many of these ECM molecules are glial-associated, developmentally regulated, and prominent in transient boundary regions during embryonic and early postnatal development. These boundary molecules were originally described on the basis of lectin-

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binding carbohydrate moieties (Cooper and Steindler, 1986a; Steindler and Cooper, 1987), and have since been shown to include the astrocyte-derived glycoprotein tenascin (Steindler et al., 1989a), as well as certain chondroitin sulfate proteoglycans (Steindler et al., 1990). Inferring from descriptive developmental studies, it appears as though these boundary molecules may exert an inhibitory effect on at least some neurites. For example, in the developing somatosensory cortical barrelfield, where individual 'whisker barrels' are delineated by these boundary molecules (Cooper and Steindler, 1986a; Steindler, 1993), many neurons extend processes that are restricted to the center of the barrel and do not cross the boundaries (Woolsey, 1990). Likewise, in the developing striatum, where boundary molecule-rich regions are present at the patch/matrix interface of the striatal mosaic, medium spiny neurons respect the boundaries and do not cross from one compartment to another (O'Brien et al., 1992). However, in vitro choice assays using dissociated cells and comparing ECM substrates to known permissive substrates have yielded conflicting results; some boundary molecule substrates appear inhibitory while others seem to promote neurite growth (for review, see Faissner and Schachner (1994) and Faissner and Steindler (1995)). Additionally, in some assays, boundary substrates seem to present a barrier to one cell type, while having no detectable effect on others (Carpenter et al., 1994; Letourneau et al., 1994). Finally, the same molecule can exhibit growth promoting and growth inhibitory properties depending on how it is presented to the cell (i.e. soluble vs. substrate-bound (Lochter et al. (1991)). In addition to inhibitory molecules, CNS lesions also result in the upregulation of trophic factors that enhance cell survival and process outgrowth (Brodkey et al., 1993; Nieto-Sampedro, 1988; Norenberg, 1994), and it is likely that the complex interaction between all of these lesion-associated molecules within the extracellular mileu is what ultimately determines the relative success or failure of regenerative growth.

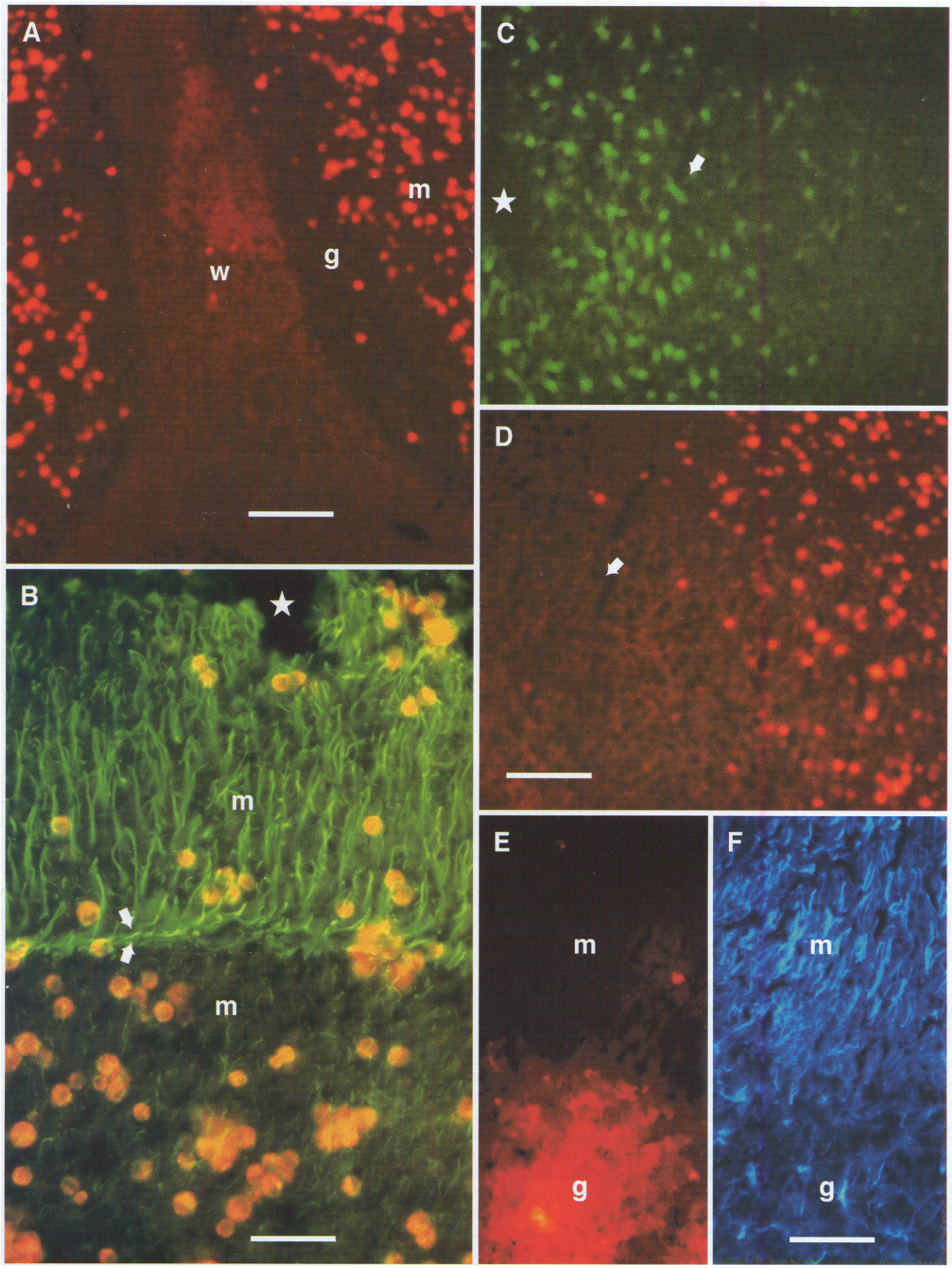
In an attempt to approximate the complex molecular environment associated with lesions in vivo (for review see Brodkey et al. (1993)), and to test the affect of lesioned-induced ECM molecules on the attachment of PC12 cells and dissociated primary embryonic neurons, we used frozen sections of lesioned adult CNS as culture substrates. Patterns of cell attachment to lesioned substrates were determined for control cultures as well as for cultures containing either polyclonal antibodies to tenascin, a glycoprotein that has been shown to be upregulated in lesioned adult CNS (Laywell et al., 1992) or a 'cocktail' of the lectins peanut agglutinin (PNA) and tetragonolobus purpureas agglutinin (TPA). We chose these two lectins because our previous studies, using lectin cytochemistry, showed that they bind to galactosyl- and fucosyl-containing glycoconjugates that outline boundaries or 'cordones' around developing functional units in the brain (e.g. whisker barrels, neostriatal striosomes, brainstem nuclei (Cooper and Steindler, 1986a; Steindler and Cooper, 1987; Steindler et al., 1989, 1990)). PNA binds to galactosyl-containing glycoconjugates that may include certain chondroitin sulfate proteoglycans which may be inhibitory to neurite growth (Crossin et al., 1989, 1990; Steindler et al., 1990), while TPA binds to fucosyl residues that could be present in a variety of boundary and wound molecules including tenascin (which is heavily fucosylated, see Kruse et al. (1985) and Steindler et al. (1990, 1995)). Whereas cells in untreated cultures avoided attaching to the substrate immediately around the wound site, addition of tenascin antibodies or the lectin cocktail to the culture medium greatly accentuated cell attachment to lesioned areas. These findings suggest that wound cryocultures can be used to study the complex ECM environment of the lesioned CNS, and that certain wound-related extracellular moieties contribute to inhibitory properties associated with CNS lesions; furthermore, neutralization of lesion-associated ECM molecules with antibodies or lectins can significantly alter cell-substrate interactions.

2. Materials and methods

2.1. Preparation of lesioned tissue

Adult ICR mice (Harlan) were deeply anesthetized with Avertin and secured in a stereotaxic device. The skull was exposed by a midline scalpel incision, and a dental drill was used to make a hole in the skull over the occipital cortex several millimeters lateral to the sagittal sinus for cortical lesions, or over the tectum several millimeters lateral from midline for cerebellar lesions. A hand-held 30 gauge needle was then inserted and pushed obliquely through cortex or cerebellum. After removing the needle, the skull hole was filled with bone wax, and the scalp was

Fig. 1. Attachment of dil-labeled PC12 (A–D) and VM (E and F) cells to frozen sections of normal (A) and lesioned (B–F) CNS after 24 hours in vitro. (A) PC12 cells attach to gray matter areas of unlesioned adult cerebellum, but fail to attach to white matter. (B) Adult lesioned cerebellum immunostained for GFAP (green) shows fewer PC12 cells attached to lesioned molecular layer (upper half) compared with an adjacent, unlesioned molecular layer (lower half). Arrows indicate fissure between adjacent folia, and asterisk indicates lesion site. (C,D) Two views of the same field of lesioned adult cerebral cortex immunostained for GFAP (green) and plated with PC12 cells [photographed with focus in the plane of the attached cells (C) and in the plane of the section (D)]. Area of high GFAP reactivity in C contains few attached cells in D. Arrow points to the same blood vessel in C and D, and asterisk indicates lesion site. (E,F) Two views of the same field of lesioned adult cerebellum immunostained for GFAP (blue in F) show dissociated VM cells (E) attached to granule cell layer, but not attached to lesioned molecular layer. Main lesion site is just out of this field of view. g = granule cell layer, m = molecular layer, w = white matter. Scale bars: A and D = 100 μm, B and F = 50 μm.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closed with surgical staples. For spinal cord lesions, postnatal day 10 mice were anesthetized with Avertin. Midthoracic vertebrae were exposed and a laminectomy was performed using a small drill and Dumont no. 5 forceps. The exposed spinal cord was then hemisected using a microknife.

Following a post-lesion survival interval of 3 days, animals were deeply anesthetized with Avertin and decapitated. The lesioned region was quickly dissected and frozen in OCT tissue protectant over liquid nitrogen. Frozen brains were stored for up to several weeks at −20°C, cut into 5 μm cryostat sections and attached to glass coverslips.

2.2. Preparation of dissociated neurons

Because our laboratory has previously established a protocol for the isolation and use of embryonic dopaminergic neurons in transplant and explant studies (Gates et al., 1995; Gates and Steindler, 1995), we chose to follow the same procedure in the present study. Time pregnant ICR mice were deeply anesthetized with Avertin on the 16th day of gestation (E16), and a midline abdominal incision was made to expose the uterus. Embryos were removed and placed into cold Gey's buffer (Gibco) supplemented with 0.45% glucose. Embryos were then quickly decapitated, and the brains were dissected out and placed in fresh Gey's. The ventral mesencephalon (VM) was isolated by microdissection of a wedge of tissue immediately dorsal to the mesencephalic flexure as described by Björklund et al. (1983). After removing the meninges, the VMs of several embryos were pooled in Gey's and dissociated by trituration with decreasing bore, fire-polished glass pipettes. The cells were then pelleted by centrifugation (5 min at 1000 rpm) and resuspended in RPMI medium supplemented with 15% heat inactivated horse serum, 5% heat inactivated fetal calf serum, glutamine, penicillin, and streptomycin.

2.3. Labeling and plating of cells, antibody and lectin perturbation

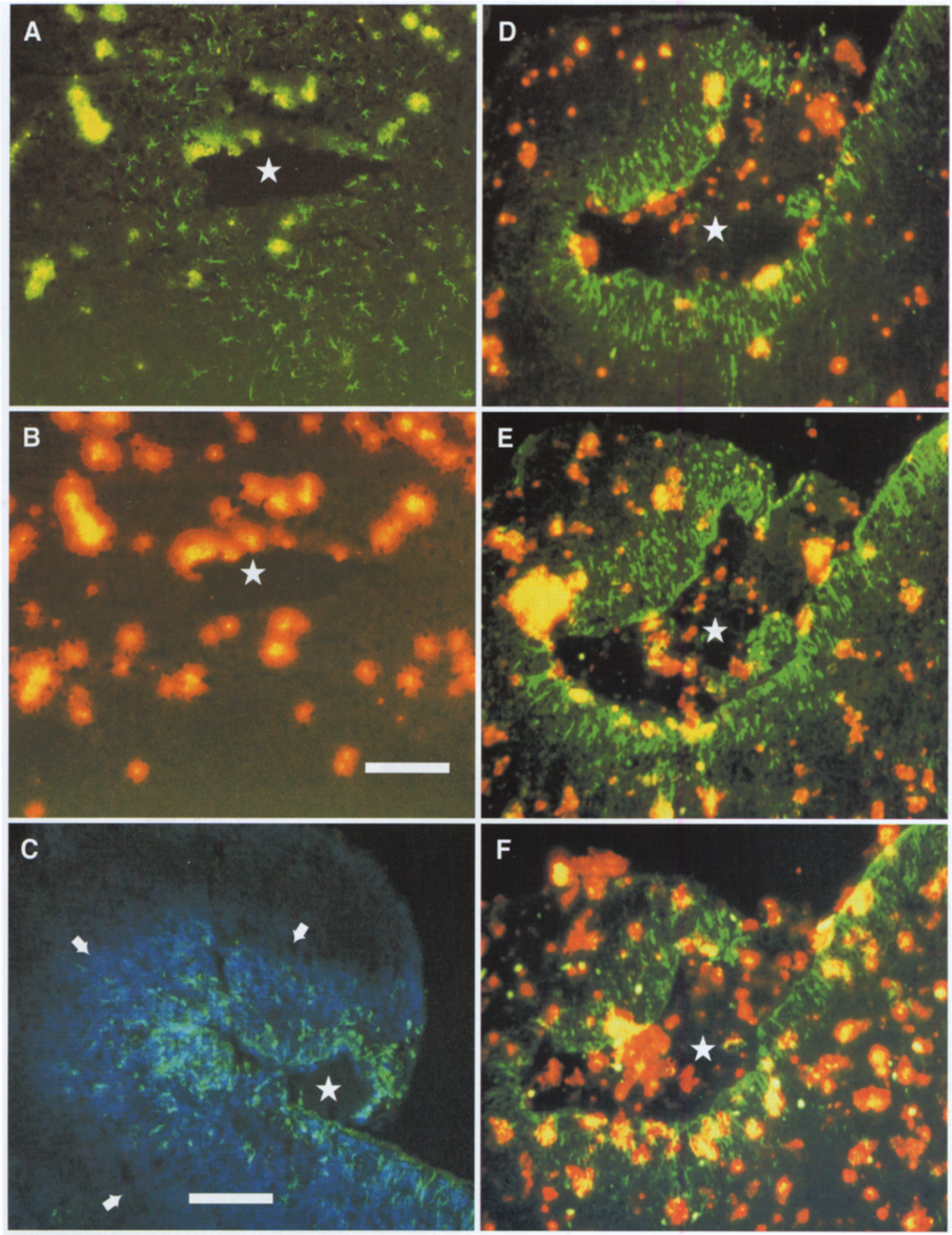
Freshly dissociated VM cells, and PC12 cells (from the rat pheochromocytoma cell line; ATCC, Baltimore) were labeled with the fluorescent carbocyanine dye, dil (Molecular Probes), according to a protocol adapted from Paramore et al. (1992). Briefly, cells were centrifuged for 10 min at 1000 rpm and resuspended in fresh medium. DiI was dissolved in absolute ethanol (2.5 mg/ml), and added to the cell suspension such that the final concentration of dil was 40 μg/ml. The cells were incubated in the dil-containing medium for 30 min at 37°C before being centrifuged and resuspended twice in fresh medium.

DiI-labeled VM and PC12 cells were triturated a final time with fire polished glass pipettes and plated onto individual glass-bound cryosections at densities ranging from 5×10^5 to 1×10^6 cells/ml in either serum-free or serum-containing medium. For each experimental run, the plating density of labeled cells was consistent between all conditions. Individual cultures were divided into experimental and control groups. Experimental cultures received media supplements of either purified lectins (galactose-binding PNA in combination with the fucose binding lotus lectin, TPA, both from Vector Labs) dialyzed against PBS and used at final concentrations of 8–32 μg/ml) or polyclonal antibodies to tenascin (Steindler et al., 1989a, 1995) at a final concentration of 0.2–10 μg/ml. Control cultures received supplements of a monoclonal antibody to the astrocyte cytoskeletal protein glial fibrillary acidic protein (GFAP), or another lectin, wheat germ agglutinin (WGA), that has to date not been found to bind significantly to CNS wound-associated molecules. Culture dishes were maintained from 2 to 24 h at 37°C in a humidified atmosphere containing 5% CO_2 and 95% O_2.

2.4. Immunocytochemistry

To prepare for immunolabeling, coverslips were removed from medium and placed in 4% paraformaldehyde at room temperature for 1–2 h. The coverslips were then rinsed in PBS and processed for immunofluorescence with a polyclonal rabbit antibody directed against GFAP (Lipshaw) to localize the lesion site. In addition, to test for preservation of wound-related ECM molecules, some cryosections were processed for immunofluorescence with a polyclonal rabbit antibody against a bacterially expressed tenascin fusion protein (a generous gift from M. Schachner, see Steindler et al. (1995) for details). Sections were

Fig. 2. Lectins and tenascin antibody alter cell attachment to lesioned cortical (A,B) and spinal cord (C–F) substrates. (A) PC12 (in this figure yellow, due to bleed through of the dil under the FITC filter) cells attach sparsely to highly GFAP-reactive (green) area surrounding an adult cortical lesion. (B) On an adjacent section treated with anti-tenascin antibody and plated at the same density, many more PC12 cells (yellow/red) attach within the lesion area (asterisk in A and B indicates lesion site). (C) Double immunofluorescence of lesioned (asterisk) P10 spinal cord shows highly reactive astrocytes (green) within an area of tenascin reactivity (blue outlined by arrows). Immunostaining for both markers is most dense at the lateral margin of this section near the wound. (D–F) Adjacent spinal cord sections treated with 0 (D), 16 (E), and 32 (F) μg/ml peanut agglutinin/lotus lectin cocktail, respectively, and plated with identical densities of PC12 cells. Notice the dose-dependent increase in cell attachment to the GFAP-reactive (green) area around the lesion (asterisk). PC12 cells, for the most part, avoid the wound area (the area most dense in GFAP + cells and dense tenascin immunostaining). In the presence of the lectin cocktail, at increasing concentrations, PC12 cells show increased attachment to both the wound area as well as neighboring 'uninvolved' white matter. The increased attachment seems to exhibit a dose-response nature, with many more dil-labeled PC12 cells attaching to the regions of intense GFAP + astrocytes. Notice in panels D–F how very few labeled cells can be seen attaching to non-tissue sites (i.e. above the sections), and that the labeled cells seen in the wound site proper are most likely attaching to wound elements and not the slide. Scale bars: 50 μm.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incubated in primary antibody overnight at 4°C, rinsed in several volumes of fresh PBS, incubated in fluorescent anti-rabbit secondary antibodies (AMCA or FITC) for 3 h at room temperature, rinsed again, coverslipped, and viewed with a Leitz epifluorescence microscope.

2.5. Cell counting procedures

Color photomicrographs of adjacent sections from a spinal cord lesion case, photodocumented in Fig. 2, were entered into a Mac II ci computer using a Hewlett Packard ScanJet IIc, and computation and analysis were performed. We currently use programs developed in our laboratory as well as the public domain NIH-Image (vol. 1.38) software. Cells from identical fields encompassing the same area of spinal cord sections (under different lectin concentration conditions) were counted using NIH Image Particle Analysis. The data was analyzed and a database established using Microsoft Excel 4.0, and graphed using Cricket Graph.

3. Results

3.1. DiI-labeled cells behave normally

In order to demonstrate that the labeling protocol does not hinder the behavior of PC12 or VM cells, we compared the ability of labeled and unlabeled cells to respond to nerve growth factor (NGF) and to white matter tracts. In both cases, labeled and unlabeled cells behaved similarly. PC12 cells have frequently been used in functional assays as surrogate neurons because they are responsive to NGF and can be induced to display neuron-like phenotypes, including the extension of long processes, when NGF is added to the culture medium (Doherty et al., 1987; Drubin et al., 1985). The diI-labeled PC12 cells used in the present study respond to NGF indistinguishably from controls by, for example, displaying a bipolar morphology and extending long 'neurites' (data not shown). Additionally, when plated onto unlesioned control sections, labeled PC12 and VM cells demonstrate a stereotypic pattern of attachment to neural tissue already extensively described; i.e., the cells readily attach to gray matter areas, but virtually never attach to white matter (Fig. 1A), except in rare instances when they are apposed to transected blood vessels. Cells exhibit this gray versus white matter difference as soon as two hours after plating, and maintain this pattern throughout the experimental protocol.

3.2. Cryopreservation of boundary molecule antigenicity

In order to demonstrate that cryosections provide a relevant injury environment during the culture protocol, unplated cryosections were incubated in culture medium before being processed for immunocytochemistry with antibodies to GFAP and the tenascin glycoprotein. After 2 days in culture, tenascin immunolabeling is detectable in cryosections within an area of intensely GFAP-reactive astrocytes indicative of a wound site (Fig. 2C). This finding is consistent with previous studies that, using fixed vibratome sections, showed an upregulation of tenascin mRNA and protein in a sub-population of astrocytes near lesions of adult rodent brain (Laywell et al., 1992). While we have not conducted a rigid time-course analysis, the fact that tenascin immunoreactivity of sections maintained 48 h in vitro closely resembles the labeling pattern seen in non-incubated sections suggests that the culture conditions do not appreciably alter the distribution or, presumably, the biological activity of tenascin during our cell attachment protocol.

3.3. Cells avoid attaching to lesion sites

When plated onto frozen sections of lesioned adult CNS, both VM and PC12 cells demonstrate a clear preference for attachment to gray matter areas not immediately associated with the lesion site. Lesion sites, as indicated by high levels of GFAP-reactive astrocytes, contain some attached cells, but many more cells attach to the gray matter just outside the wound area. This pattern is consistent in lesioned tissue from cortex (Fig. 1C,D), cerebellum (Fig. 1B), and spinal cord (Fig. 2D–F). In some cases the contrast between attachment to lesion and non-lesion areas is quite striking, with a sharply demarcated line of cell

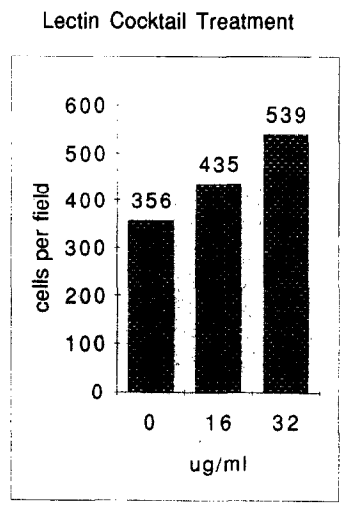

Fig. 3. Histogram of cell counts from di-labeled PC12 cells plated on adjacent spinal cord wound sections (as shown in Fig. 2D–F), cultured for 1 day, counted and plotted as described in section 2 confirms larger numbers of labeled PC12 cells (counted as diI-labeled 'particles') attach, in a dose-response manner, to these sections (including wound sites) in the presence of higher concentrations of the lectin cocktail. Counts were made from identical whole fields (slightly larger than those shown in Fig. 2D–F) of these serial 8 μm cryosections through the same wound, with the same number of labeled PC12 cells plated onto each of the sections. The exact numbers of labeled PC12 cells or particles attaching to the section in 'B' is 356, in 'C' 435, and to 'D', 539.

attachment corresponding to a definite border between areas of high and low GFAP immunoreactivity (compare Fig. 1C and D, and E and F). In other cases, the pattern of attachment between lesioned and non-lesioned tissue is much more gradual. In cerebellar lesions, for instance, the highly reactive molecular layer involved in the wound supports some cell attachment, but the molecular layer of an adjacent folium supports much greater cell attachment (Fig. 1B).

3.4. Lectins and tenascin antibodies facilitate cell attachment to lesions

Addition of purified lectins or anti-tenascin antibody to the culture medium results in a significant enhancement of cell attachment to lesioned areas. In comparison to identically plated controls, cultures that received media supplements of anti-tenascin antibodies or the lectin cocktail show significantly more cell attachment within the highly GFAP-reactive area around the wound (Fig. 2). Fig. 3 shows the dose-response nature of the lectin cocktail effect on PC12 cell attachment to adjacent cryosections of lesioned spinal cord; more cells attach to the wound areas, as well as surrounding intact spinal cord tissue that is primarily white matter at these levels.

4. Discussion

Frozen tissue sections have the advantage of preserving many of the proteins present in vivo in an easily accessed, two-dimensional structure. Previous studies have used frozen tissue section culture substrates in a variety of assays to examine neurite growth (Carbonetto et al., 1987; Covault et al., 1987; Crutcher, 1989; Crutcher and Privitera, 1989; Carpenter et al., 1994; Schwab et al., 1993; Fredericks et al., 1993; Geisert, 1991; Sagot et al., 1991; Sandrock and Matthew, 1987; Savio and Schwab, 1989; Tuttle and Matthew, 1991; Watanabe and Murakami, 1989). For example, this method was used in a study of the neurite outgrowth pattern of ciliary ganglion neurons on frozen sections of innervated versus denervated muscle (Covault et al., 1987) which showed that denervated muscle seemed to support more growth than normal, innervated muscle. Likewise, frozen sections were used to examine differential neurite outgrowth between cells provided with CNS gray versus white matter substrates (Crutcher, 1989; Sagot et al., 1991; Savio and Schwab, 1989), and between cells provided with PNS versus CNS substrates (Carbonetto et al., 1987; Sandrock and Matthew, 1987). This technique has been used to demonstrate that neurons, for the most part, progressively lose the ability to extend neurites on CNS white matter as they mature (Shewan et al., 1995), and that CNS gray matter loses its ability to stimulate neurite outgrowth with increasing age (Sagot et al., 1991). Thus, there seems to be a consensus from previous cryoculture assay studies that: (1) gray matter supports neuronal attachment and growth better than white matter; (2) sciatic nerve is generally more favorable to attachment and growth than optic nerve which is about as repulsive as adult brain and spinal cord white matter (except see Geisert and Stewart (1991) who showed that embryonic cortical neurons were supported better on sections of adult cortex when compared to optic nerve which was even better than sections of sciatic nerve); and (3) attachment and growth seems to be age dependent; i.e., the younger the cells plated and the younger the cryosection substrate, the better. However, Shewan et al., 1995, found that young dorsal root ganglion neurons seem unresponsive to the presumed inhibitory environment of mature optic nerve cryosections, and these investigators suggested that these young cells may not express receptors for the so-called growth-repulsive molecules. None of the aforementioned studies used sections of injured adult brain for their cryoculture assays, but some of the previous studies did attempt to perturbate the molecular environment of their growth assays (see below).

Cryosections of adult mouse cerebellum yielded cell attachment patterns with labeled PC12 cells that were identical to that seen using neuroblastoma cells on adult rodent cerebellar sections (Fig. 1A) (Savio and Schwab, 1989). The use of dil labeling of cells prior to culturing proved to be extremely effective, compared to previously used markers, in allowing the recognition of distinct patterns of distribution of both PC12 and embryonic ventral mesencephalon cells on both normal and lesioned tissue sections. Zwimpfer and his collaborators (Zwimpfer et al., 1992) have reported that, following the autografting of optic nerve to the adult rodent cerebellum, axonal growth from the nerve seemed to preferentially occur in the cerebellar granule cell layer as opposed to the molecular layer. Lesions of the cerebellum result in abundant expressions of both messenger RNA and protein for putative inhibitory ECM molecules such as tenascin (Laywell et al., 1992), and it seems apparent that the nerve grafting experiments described above would also result in lesions that lead to the upregulation of these molecules. The present study has shown that lesions of the cerebellum also yielded more PC12 and neuronal attachment to the granule cell layer than the overlying molecular layer (see Fig. 1E,F), and it was consistently and dramatically demonstrated that cultured cells would avoid one molecular layer that contained an extensive reactive astrogliosis while attaching to an adjacent folial molecular layer that was not involved in the lesion (see Fig. 1B). The present study has thus shown that frozen sections of lesioned adult CNS tissue can be used to study the interaction of paraneuronal cell lines and neuronal cells with lesion-associated extracellular matrix proteins. Furthermore, this system provides a simple way to test the effects of functional blocking of these proteins with lectins or antibodies. Significantly, the function-blocking effects seen in the present study demonstrate a

dose-dependent response for the concentrations examined. Previous studies using 'molecular perturbation' in cryoculture assays have utilized: addition of growth factors or antibodies to growth factors (i.e. nerve growth factor, NGF) to alter the attachment and growth of superior cervical ganglia explants on cryosections of sciatic nerve (Sandrock and Matthew, 1987); antibodies to putative myelin-associated inhibitory proteins to augment the growth of neuroblastoma cells on CNS white matter (Savio and Schwab, 1989); macrophages or macrophage-conditioned media on cryosections of adult rat optic nerve (David et al., 1990); as well as early postnatal rat cortical glial-conditioned media to augment the attachment and growth of embryonic hippocampal neurons on cryosections of adult CNS (Carpenter et al., 1994). All of these approaches generated impressive increases (or in the case of antibodies to NGF, decreases) in the attachment and/or growth of cultured cells on 'normal' CNS or PNS cryosections. To our knowledge, the present study is the first demonstration of accentuated attachment of cells to cryolesions using either antibodies or lectins that bind to putative growth-inhibitory moieties associated with adult CNS wounds. The proven ability of function-blocking tenascin antibodies to interfere with cell migration in vitro (Husmann et al., 1992), and the demonstration that PNA can negate the inhibitory components of the ECM during axon pathfinding events (Oakley and Tosney, 1991) add credence to the present findings that such interventions also facilitate cell attachment to lesioned CNS. The lectin cocktail findings presented here are particularly worthy of further experimentation. The use of these two lectins (versus WGA) in a cocktail on spinal cord cryolesions greatly facilitated the attachment of PC12 cells, in an apparent dose-response nature, and future studies need to determine whether this is a specific effect mediated by the binding of these lectins to their respective sugar moieties present in the wound (perhaps using hapten sugar competition studies), or whether these divalent lectins may have other effects on the attachment of cells on wounds including charge effects or cross-linking of the cultured cells to the substrate.

The present results, while suggesting that blocking lesion-associated macromolecules can make reactive areas of the CNS more favorable for cell attachment, do not address the issue of neurite extension or regeneration. It is known that neuritic growth is dependent on a favorably adhesive substrate (for review, see Bixby and Harris (1991), Faissner and Schachner (1994), Faissner and Steindler (1995), Reichardt and Tomaselli (1991) and Sanes (1989)), but adhesiveness alone is not sufficient to produce neurite extension. Indeed, preliminary work from our laboratory has shown that some lesion-associated matrix molecules can promote cell adhesion while concomitantly retarding neurite outgrowth (Gates and Steindler, 1995). Plating primary dissociated neurons on wound substrates and analyzing neurite outgrowth properties under control and blocking conditions would, perhaps, be the best approach to elucidate the effects of wound-related molecules on growing neurites. Besides the quantitative particle analysis performed here, to quantify the numbers of cells attaching to normal or lesioned cryosections under so-called neutralizing conditions, there are other methods that can be used to measure the effects of molecular perturbation of putative inhibitory molecules. Using a 'transept approach' to perform cell counts and avoid sample bias (Richfield et al., 1995), a transept can be placed along mediolateral, rostrocaudal, or dorsoventral fields that include or do not include the center of a given lesion. It is also possible to use a modified Sholl analysis (Greenough and Chang, 1988; Hubener and Bolz, 1992; Sholl, 1953), which also can be performed on each wound or neutralized wound studied, with concentric circles of increasing radius drawn around the center of each wound, and determining the angular locations in relation to the zero degree axis of wound intersections with the circles. Using this method and establishing a large database, one would be able to quantitatively describe the configuration of cell attachment, or neurite growth from attached cells in relation to distances from the center of a wound where it is assumed that the highest concentrations of growth-inhibitory substances are expressed in comparison to penumbral areas (Brodkey et al., 1993; Nieto-Sampedro, 1988).

While the frozen sections are a favorable substrate for neurons and PC12 cells, and we have shown that lesion-associated ECM molecules are immunologically preserved, there are caveats to be aware of with this assay. Most significantly, frozen sections may lose biological activity in storage, although studies have shown that many susceptible enzymes are in fact preserved for extensive periods of time in cryosections of, for example, liver (Fredericks et al., 1993). However, a progressive loss of biological activity may occur with many other proteins, and there may not necessarily be a concomitant loss of immunoreactivity. This being the case, it seems reasonable to use relatively fresh tissue for these in vitro assays.

In conclusion, the present study has shown that cryolesion cultures and PC12 cells offer a useful assay for studies of growth-associated molecules in CNS wounds. PC12 cells offer many advantages over other cell lines as well as primary cultures not only because of their hardiness, but also because there is a significant amount known about their cell biology including mechanisms of their neuritic growth (e.g. see Doherty et al. (1987) and Drubin et al. (1985)). The cryolesion-in-a-dish assay offers an alternative approach, with both advantages and disadvantages, to previous scar-in-a-dish assays (David et al., 1990; Rudge and Silver, 1990; Rudge et al., 1989) of cell and molecular interactions during CNS wounding and regenerative attempts. One disadvantage to the cryolesion model is that plated cells are exposed not only to cut membranes, but also to organelles and intracellular molecules that they may or may not encounter in vivo. As advantages, the

cryolesion model affords the opportunity to disregard active tissue processes including the complex remodeling (including debris removal) that occurs in living scar explants (Gates and Steindler, 1995), is more than a cellular lattice out-of-context that typifies the nitrocellulose-harvested, reactive astrocytes of the previous scar-in-a-dish model (Rudge and Silver, 1990; Rudge et al., 1989), and it allows one to preserve a wound in a suspended state of animation for identifying discrete aspects of cell-substrate interactions.

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